CHROMATOGRAPHY OF PROTEINS AND PEPTIDES ON SEPHADEX ION-EXCHANGERS: DEPENDENCE OF THE RESOLUTION ON THE ELUTION SCHEDULE

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1. Introduction

Ion-exchangers with cross-linked dextran gel matrix [1] have found extensive use in the fractionation of protein and peptide mixtures. Proteins and peptides very strongly interact with ion-exchangers; most frequently, they are either completely adsorbed ($K_d = \infty$; $R_f = 0$) or not adsorbed at all ($K_d = 0$; $R_f = 1$) [2], depending on the pH and ionic strength of the solution. Finite distribution coefficients are seldom obtained and only in well specified conditions. Consequently, chromatographic theories operating with finite values of distribution coefficients [3] cannot be used as guides for experimentation. Instead, empirically found conditions of gradient elution [4, 5] are used for successive elution of mixture components from the ion-exchanger.

In this paper, general conclusions are drawn from extensive experiments with a linear gradient of molarity of a neutral salt (ionic strength gradient). The result is a simple rule permitting the determination of the column volume and volume and slope of the gradient which are the most suitable for the fractionation of a given amount of a certain mixture.

2. Experimental

The conclusions presented in this paper are based on experiments in which either pig immunoglobulin polypeptide chains or peptides from tryptic digest of pig immunoglobulin κ chains were fractionated on SE-(sulfoethyl)-Sephadex C-25 and QAE-(quaternized aminoethyl)-Sephadex A-25 [6, 7]. The following buffers were used: chromatography on SE-Sephadex: 5 mM potassium formate, 8 M in urea, adjusted to pH 3.0 with formic acid; chromatography on QAE-Sephadex: 0.01 M sodium acetate, 8 M in urea, adjusted to pH 5.0 with acetic acid. The ionic strength gradient was produced by addition of a neutral salt (potassium or sodium chloride) to the buffer (see fig. 1 for further details).

3. Theoretical

It has been known that the actual results of fractionation of a certain mixture can differ according to the arrangement of the experiment even when the fundamental conditions (type of ion-exchanger, buffer) remain unaltered.

The resolution strongly depends on the volumes in which the peptide peaks emerge from the column. Peptides emerging in too large volumes (i.e. at a low concentration) yield flat, undistinct peaks, difficult to evaluate. In contrast, if the peptides emerge in volumes which are too small, the resolution is also impaired. It follows therefore that there is a certain range of optimum values of column volume and gradient volume for the fractionation of a certain amount of a given mixture.

Considering the influence of the arrangement of the experiment on the resolution, following symbols will be used:

V_{pep} — Volume of buffer in which the peptide emerged from the column (cf. fig. 1)

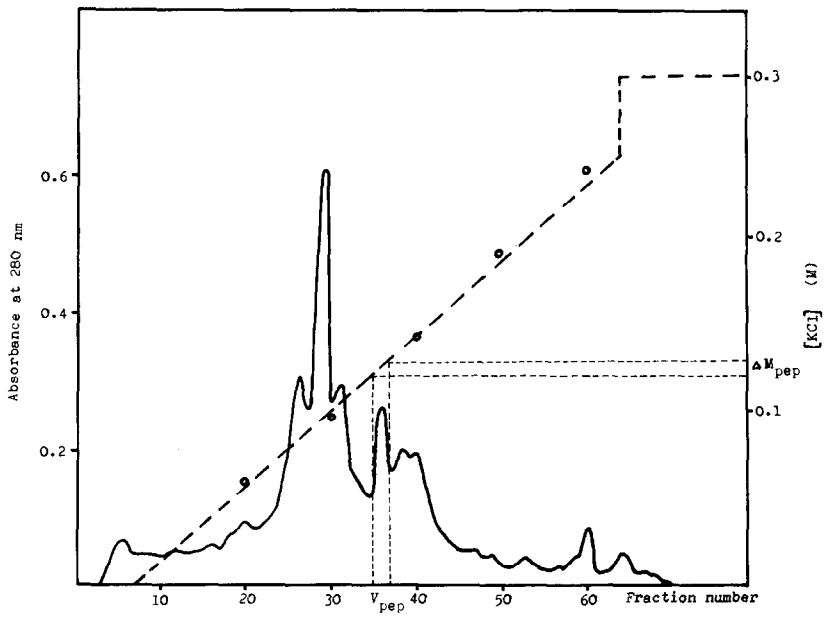

Fig. 1. Fractionation of peptides from tryptic digest of pig immunoglobulin κ chains on SE-Sephadex C-25 by linear molarity gradient of potassium chloride. The column (1.2 × 20 cm) was equilibrated with 5 mM formate buffer pH 3.0, containing 8 M urea. After the application of sample (20 mg in 2 ml of buffer) the column was eluted by a linear gradient of potassium chloride in the buffer at a rate of 8.5 ml/cm^2/hr. The mixer contained 100 ml of buffer and the reservoir 100 ml of buffer containing 0.25 M potassium chloride. After gradient elution had been terminated, the column was washed with buffer containing 0.30 M potassium chloride. Peptides from pooled fractions were freed of urea by the passage through the column of Sephadex G-25 equilibrated with 0.2% formic acid and freeze-dried. —— absorbance at 280 nm, - - - - molarity of potassium chloride. See text for explanation of V_{pep} and ΔM_{pep}.

ΔM_{pep} – molarity range in which the peptide emerged from the column (cf. fig. 1)

V_{grad} – gradient volume, i.e. volume of buffer in the mixer plus volume of buffer in the reservoir

ΔM_{grad} – range of gradient molarity, i.e. difference (molarity of neutral salt in the reservoir minus molarity of neutral salt in the mixer) at the beginning of the experiment

V_{col} – column volume

ΔM_{col} – molarity range in the column, i.e. difference (molarity of neutral salt in the head of the column minus molarity of neutral salt at the outlet of the column)

$S = \frac{\Delta M_{grad}}{V_{grad}} = \frac{\Delta M_{col}}{V_{col}}$ – slope (steepness) of gradient molarity

$G = \frac{1}{S}$ – gradient volume normalized for molarity range 0.00–1.00 M

As demonstrated in fig. 2, it is the molarity range ΔM_{pep} that remains constant if a peptide is chromatographed at molarity gradients of varying steepness. Hence a peptide emerges at a volume V_{pep} which corresponds to the molarity range ΔM_{pep}. Volume V_{pep} increases with increasing gradient volume V_{grad} for the same gradient range ΔM_{grad}. Similarly, volume V_{pep} increases with decreasing range ΔM_{grad} for the same volume V_{grad}. The same relation exists between volume V_{pep} and molarity range in the column ΔM_{col}. Thus, e.g. for the same range ΔM_{col}, volume V_{pep} increases with increasing column volume V_{col}.

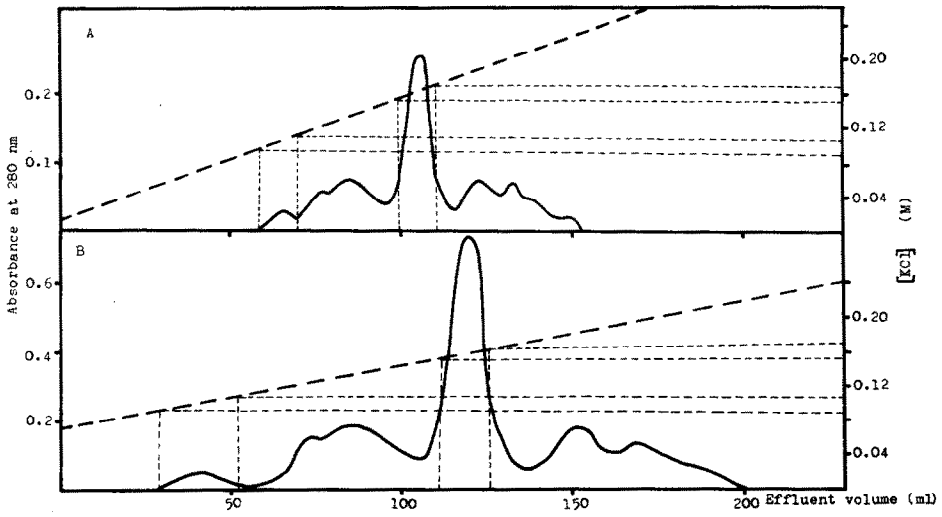

Fig. 2. Fractionation of peptides from tryptic digest of pig immunoglobulin κ chains on SE-Sephadex C-25 at pH 3.0. In both runs, the same peptide mixture was fractionated. The column and gradient parameters; (A) V_{col} = 22 ml, G = 800 ml (V_{grad} = 200 ml, ΔM_{grad} = 0.25), $\Delta M_{col} = 2.7 \times 10^{-2}$; (B) V_{col} = 36 ml, G = 1600 ml (V_{grad} = 300 ml, ΔM_{grad} = 0.19), $\Delta M_{col} = 2.3 \times 10^{-2}$. The volumes in which peptides emerged from the column in A and B were 12 and 24 ml, respectively. These volumes correspond to the molarity range $\Delta M_{pep} = 1.5 \times 10^{-2}$. —— absorbance at 280 nm, - - - - molarity of potassium chloride. See text for explanation of symbols.

From what has been said:

$$\frac{V_{pep}}{V_{col}} = \frac{\Delta M_{pep}}{\Delta M_{col}} \quad (1)$$

and also

$$\frac{V_{grad}}{V_{col}} = \frac{\Delta M_{grad}}{\Delta M_{col}} \quad (2)$$

It follows therefore that

$$V_{pep} = \Delta M_{pep} \left[\frac{V_{grad}}{\Delta M_{grad}}\right] \quad (3)$$

and also that

$$V_{pep} = \Delta M_{pep} \left[\frac{V_{col}}{\Delta M_{col}}\right] \quad (4)$$

Using the defined relation for normalized gradient volume,

$$V_{pep} = \Delta M_{pep}\, G \quad (5)$$

and

$$V_{pep} = \frac{\Delta M_{pep}}{S} \quad (6)$$

4. Implications and discussion

The relations derived can be used in practice to solve a common separation problem, namely, what should be the column volume (V_{col}) and gradient slope (i.e. V_{grad} and ΔM_{grad}) for the optimal resolution of a given amount of a protein mixture provided that other conditions (type of ion-exchanger, pH, buffer) are given. In other words, experimental conditions are sought at which the components are eluted at concentrations neither extremely low nor extremely high. Two different approaches are possible:

(i) The first one concerns cases in which the exact composition (qualitative and quantitative) of the protein mixture is known. Thus, the optimal concentration at which a certain component should be eluted from the column can be chosen precisely. Thus,

its V_{pep} value is fixed and if the ΔM_{pep} value is known (vide infra) the G value can be calculated from (5); in this way, the gradient parameters are found. Provided that the ΔM_{col} value is known (vide infra) the V_{col} value can be calculated from (4).

(ii) The second approach concerns cases in which the composition of the protein mixture is unknown but can be roughly estimated. In these cases, the ΔM_{col} and G values cannot be exactly calculated; however, they can be approximated from previous experience. Thus, e.g. in the experiment described in fig. 1, the ΔM_{col} and G values were 2.8×10^{-2} and 8×10^{2}, respectively. The ratio (weight of peptide mixture/column volume) was roughly 1 mg/ml. According to experience in our laboratory, if the same mixture is to be fractionated at the same ΔM_{col} value and at the ratio (weight of peptide mixture/column volume) = 10 mg/ml, the G value should be approximately 8×10^{3}.

For both approaches, the experimentally checked values of ΔM_{pep} and ΔM_{col} are necessary. According to our experience, the value of ΔM_{pep} equals approximately 1.5×10^{-2} (i.e. the peak of the peptide emerges in the molarity range 0.015M e.g. between 1.12 and 0.135 M KCl). The range of suitable values of ΔM_{col} is 2.5×10^{-2} to 4.0×10^{-2}. It should be noted that these values are derived from experiments with buffers, 8 M in urea. In other experimental conditions, the empirically derived values of ΔM_{pep} and ΔM_{col} might not equal those given in this paper.

The value of ΔM_{pep} depends considerably on the set-up of the column outlet and tubing through which the effluent passes into individual fractions. If the diameter of the column outlet or of the tubing is too large, the peak of the peptide is blurred and emerges at a volume larger than that in which it was originally eluted.

It is often supposed that an imperfect fractionation can be improved by using a shallower gradient (i.e. higher G value). From what has been said above, however, it follows that this is true only when proteins are eluted from the ion-exchange column at high concentrations (of about 10^{-3} M and higher). Much more often the proteins are eluted at relatively low concentrations (approximately 10^{-4} M and lower). In this case, on the contrary, the fractionation can be improved by using a steeper gradient (i.e. lower G value).

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